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The Domain Structure of Centromeres Is Conserved from Fission Yeast to Humans

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The centromeric DNA of fission yeast is arranged with a central core flanked by repeated sequences. The centromere-associated proteins, Mis6p and Cnp1p (SpCENP-A), associate exclusively with central core DNA, whereas the Swi6 protein binds the surrounding repeats. Here, electron microscopy and immunofluorescence light microscopy reveal that the central core and flanking regions occupy distinct positions within a heterochromatic domain. An "anchor" structure containing the Ndc80 protein resides between this heterochromatic domain and the spindle pole body. The organization of centromere-associated proteins in fission yeast is reminiscent of the multilayered structures of human kinetochores, indicating that such domain structure is conserved in eukaryotes.

INTRODUCTION

Centromere function requires the proper orchestration of several subfunctions, such as kinetochore assembly, sister chromatid cohesion, binding of kinetochore microtubules, orientation of sister kinetochores to opposite poles, and their movement toward the spindle poles. Centromere structure may be organized so as to accomplish these functions in different, separable domains. Although centromere functions have been scrutinized in several genetically tractable model organisms, such as *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe,* and *Drosophila melanogaster*, detailed structural studies have been limited or lacking in all these organisms. The most comprehensive molecular view of a centromere is available in *Saccharomyces cerevisiae*. The "point centromeres" of this organism are built on a single nucleosome with what contains the histone H3 variant CSE4 (CENP-A), the CBF3 DNA-binding complex, and 12 additional proteins already identified (Pluta *et al.*, 1995; Pidoux and Allshire, 2000). However, the compact size of these centromeres (125 bp) renders them too small for fine structural analysis. The "regional" centromeres of *Drosophila* (420 kb) are more typical of the centromeres found in the vast majority of eukaryotes (Murphy and Karpen, 1995). Here, the DNA is quite well characterized (Sun *et al.*, 1997) and the centromeric DNA has been shown to display an epigenetic structure (reviewed by Karpen and Allshire, 1997). *Drosophila* kinetochores also appear bilaminar by electron microscopy (EM; Goldstein, 1981), but the positions of centromere-binding proteins within these structures have not been determined. In contrast, the centromeric DNA of humans is not well understood, but the fine structure of their kinetochores has been studied extensively, particularly through the binding of autoantibodies from human patients with scleroderma. These immunoglobulins react with several distinct centromere proteins (CENPs; Brenner *et al.*, 1981; Earnshaw and Migeon, 1985; Earnshaw and Rothfield, 1985). As seen by EM, the human metaphase centromere is multilayered and contains several substructures: a fibrous corona, an outer and inner plate, and the space between them. Underlying these plates is the heterochromatic region that underlies the inner plate (reviewed by Pluta *et al.*, 1995). Each of these substructures appears to comprise a distinct protein composition. The fibrous corona contains CENP-E, dynein, and dynactin, the outer CENP-F, the inner plate contains CENP-C and CENP-A, and the underlying heterochromatin contains CENP-B, INCENP, HP1, and Suvar3–9 (Saitoh *et al.*, 1992; Cooke *et al.*, 1997, 1990; Vafa and Sullivan, 1997; Warburton *et al.*, 1997; Yao *et al.*, 1997; Aagaard *et al.*, 1999).

In fission yeast, the centromere DNA has been functionally defined (reviewed by Pidoux and Allshire, 2000). Fission yeast centromeres occupy 40–100 kb on the chromosome and all three have a symmetric organization. A central core sequence (*CC/cnt*) is flanked by arrays of repeated

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(inner *imr/B* and outer *otr/K+L*) sequences (Clarke *et al.*, 1986; Chikashige *et al.*, 1989; Clarke and Baum, 1990). These chromosomal elements also show clear epigenetic structure (Steiner and Clarke, 1994; Ekwall *et al.*, 1997). There is no similarity between sequences of centromeric DNA in *S. pombe, S. cerevisiae, Drosophila* and humans, but on the basis of their size and organization, *S. pombe* centromeres can be classified as "regional" (Pluta *et al.*, 1995). In *S. pombe* several CENPs have been identified: Swi6, Chp1, Cnp1, Mis6, Mis12, Ndc80, Nuf2, and Spc24 (Ekwall *et al.*, 1995; Saitoh *et al.*, 1997; Doe *et al.*, 1998; Goshima *et al.*, 1999; Takahashi *et al.*, 2000) (Wigge and Kilmartin, 2001). Chromatin immunoprecipitation cross-linking experiments have demonstrated that Cnp1 (*S. pombe* CENP-A) and Mis6 proteins both bind to the central core region but not the flanking regions. Conversely, the chromodomain proteins Swi6 and Chp1 bind the flanking repeats but not the central core region. This indicates that there are two distinct structural and functional domains in *S. pombe* centromeres (Partridge *et al.*, 2000) (Goshima *et al.*, 1999; Saitoh *et al.*, 1997; Takahashi *et al.*, 2000; see Figure 1A).

In the work reported here we are exploring the organization of centromere-binding proteins in fission yeast during interphase. Studies by immunofluorescence in vertebrates have shown that several such proteins are localized during interphase as if they were still associated with centromeric DNA, e.g., CENP-A, -B, and -C (Pudenko *et al.*, 1997), whereas others are not, e.g., CENP-E (Cooke *et al.*, 1997). The difficulty of studying the organization of centromere-binding proteins during interphase in vertebrates is that neither the position nor the orientation of these structures appears to be controlled at this cell cycle time. In fission yeast, on the other hand, the centromeres are localized to a specific part of the nucleus that lies immediately beneath the nuclear envelope, opposite the spindle pole body (SPB; which is situated in the cytoplasm; Funabiki *et al.*, 1993; Ekwall *et al.*, 1995; Ding *et al.*, 1997). This situation makes it possible to know approximately where all the centromere-binding proteins will be positioned and to measure their relative positions with reference to the SPB. In this study we used light and electron microscopic immunolocalization to find these relative positions and to determine whether the centromere-binding proteins are organized during interphase. Our evidence implies that these proteins are ordered and that their order can be related to the part of the centromere to which they bind. We infer that the portion of the fission yeast chromosome that is essential for normal segregation at mitosis is rigorously positioned during interphase, both before and after SPB replication. Despite the differences in their DNA sequences, the centromeres of fission yeast appear similar in their design to those of humans, suggesting that a multilayered organization may be conserved in many eukaryotes. The functional implications of this observation are discussed.

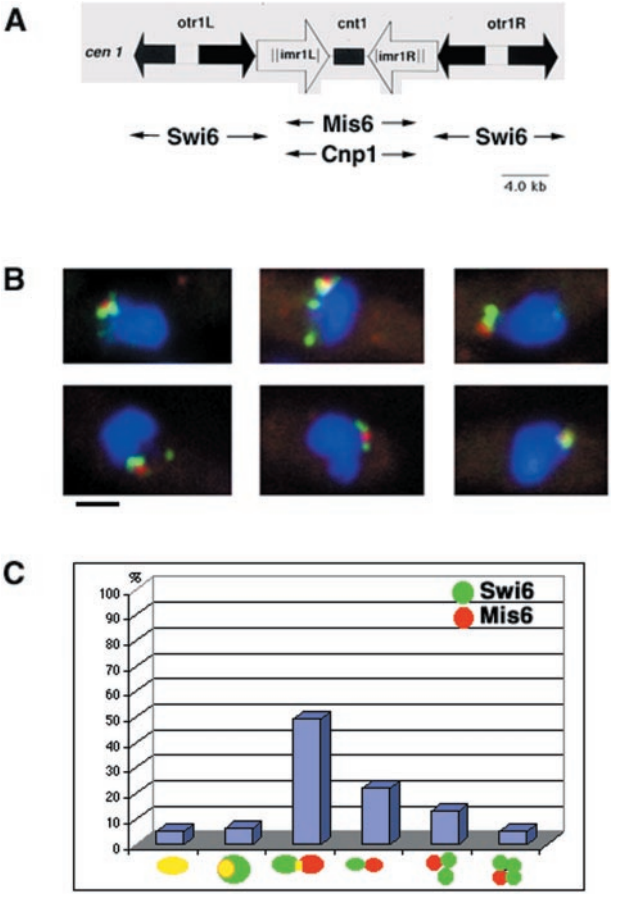

Figure 1. The central core and flanking regions are cytologically distinct. (A) Schematic representation of fission yeast centromere structure and binding proteins. The schematic structure of *cen1* is depicted together with the chromatin cross-linking chromatin immunoprecipitation data indicating where Swi6, Mis6 (Partridge *et al.*, 2000), and Cnp1 proteins (Takashi *et al.*, 2000) bind to the centromere DNA. (B) IF pictures showing the localization of Swi6 (green) and Mis6-HA (red) proteins in interphase nuclei. Swi6 binds to all heterochromatic regions in *S. pombe* nuclei, i.e., centromeres, telomeres, and *mat2/3* regions, but the major signal in interphase cells represents the clustered centromeres (Ekwall *et al.*, 1995). DAPI (blue) was used to stain the DNA. Bar, 2.0 μm. (C) Statistical analysis of the relative positions of Swi6 and Mis6 at centromeres. Yellow color indicates colocalization in the merged picture (n = 100).

MATERIALS AND METHODS

S. pombe strains carrying the markers *mis6-3xHA-LEU2*$^+$ (Saitoh *et al.*, 1997), *cut12-Pk-ura4*$^+$ (Bridge *et al.*, 1998), and *ndc80-GFP-kanMX6* (Wigge and Kilmartin, 2001) were prepared for immunofluorescence microscopy (IF) by the formaldehyde fixation procedure (Hagan and Hyams, 1988) with some modifications. Log-phase cultures were incubated for 5–30 min in YES + 1.2 M sorbitol before harvest. PEMAL (PEM + 5 or 0.03% milk, 0.1 M L-lysine HCl, cleared by centrifugation during 30 min at 20,000 × *g*) was used instead of PEMBAL. Primary antibodies were mouse anti-hemagglutinin (HA; Boehringer and Mannheim, Indianapolis, IN), mouse anti-Pk (Serotec, Oxford, UK), rabbit anti-green fluorescent protein (GFP; Molecular Probes, Eugene, OR), rabbit anti-Swi6 (Ekwall *et al.*, 1995), and sheep anti-Cnp1 (Mellone and Allshire, unpublished data). Fluorescein isothiocyanate or Texas Red-conjugated secondary antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA) or Sigma (St. Louis, MO). Cells were visualized with the use of an Axioskop II microscope (Zeiss, Oberkochen, Germany) equipped with a C4742–95 charge-coupled device camera (Hamamatsu, Middlesex, NJ). A 100× objective lens

with NA 1.3 produced images with a calculated resolution of 211 nm.

For EM localization of GFP fusion proteins, samples of *S. pombe* cells harboring GFP-Swi6 (Pidoux *et al.*, 2000), GFP-Cnp1 (Mellone and Allshire, unpublished data), and Ndc80-GFP (Wigge and Kilmartin, 2001) before or after were prepared by a modification of the methods of (Ding *et al.*, 1997). In brief, *S. pombe* cells grown in liquid cultures were harvested by centrifugation and frozen in a high-pressure freezer (Balzers, Lichtenstein) with 2300 bar within 0.6–0.7 s. Frozen samples were freeze-substituted into 1% formaldehyde in methanol at −93°C for 10 h, warmed to −61°C for 6 h, warmed to −38°C for 1 h, and embedded in Lowicryl K11M. Serial sectioning was to a section thickness of 30–50 nm.

Immunostaining was carried out after blocking overnight in 0.1 M phosphate buffer, pH 7.4, with 10% bovine serum albumin or 10% donkey serum for 1.5 h and addition of rabbit antibodies to GFP (A11122, Molecular Probes) diluted 1:100 in the same buffer at 4°C. GFP fusion proteins were followed by protein A conjugated to 10-nm colloidal gold (Au_{10}) or donkey anti-rabbit antibodies conjugated to 12-nm colloidal gold (Au_{12}) for 2 h. Cells were postfixed in 2% glutaraldehyde for 15 min and poststained with uranyl acetate for 7 min and lead citrate for 4 min. The average labeling densities on the heterochromatin domains in G2 cells were 162 ± 43 $Au_{10}/\mu m^2$ for Swi6 and 13 ± 14 $Au_{10}/\mu m^2$ for Cnp1. The background staining of gold in the nucleus was 13 ± 4/μm^2 for Swi6 and <2/μm^2 for Cnp1. The nonspecific background staining in the cytoplasm was 3 ± 4 and 1 ± 2 $Au_{10}/\mu m^2$, respectively. Serial sections were imaged in a Leo906 80-kV electron microscope, the resulting EM pictures were scanned with a snapscan (Agfa, Ridgefield Park, NJ), and three-dimensional (3-D) computer models were generated with the IMOD software package (Kremer *et al.*, 1996).

RESULTS

Central Core and Flanking Domains Are Cytologically Distinct

Immunofluorescence microscopy (IF), with antibodies that recognized Swi6 and Mis6 (represented by an HA-tagged allele; Saitoh *et al.*, 1997), was carried out with unsynchronized, log-phase cultures of *S. pombe*. Swi6 localizes to all heterochromatic regions in *S. pombe* nuclei, i.e., centromeres, telomeres, and *mat2/3* regions, but the major signal in interphase cells corresponds to the centromeres, which are clustered near the SPB (Ekwall *et al.*, 1995). Double immunolabeling of Mis6-HA (red) and Swi6 (green) indicated that Swi6p and Mis6p colocalized in only a minority (11%) of cells (Figure 1, B and C). In 100 cells analyzed, 49% of the signals were partially overlapping but clearly distinct, 22% of the signals were adjacent but not overlapping, and 18% of cells showed two to three Swi6 spots surrounding a Mis6 spot (Figure 1C). In control experiments double immunolabeling of Mis6-HA (red) and Cnp1 (green) resulted in 100% colocalization (n = 50). Thus, Mis6p and Swi6p, proteins with previously described distinctions in their DNA-binding domains (Partridge *et al.*, 2000), show cytologically distinct localizations. The variability of the localizations we have seen also suggest that the relative positions of these two centromere subdomains are highly dynamic in interphase cells.

This finding prompted us to investigate the location of these CENPs relative to the newly described CENP, Ndc80 (Wigge and Kilmartin, 2001). With the use of antibodies directed against the Cnp1 protein (green) and the GFP (red), which was present as a chimera with Ndc80, we learned that these two proteins were partially colocalized in all interphase cells (Figure 2, A and B). Based on 50 cells analyzed, the Ndc80 signal was generally larger than the Cnp1 signal; in 14% of cells the Ndc80 signal protruded toward the nuclear periphery (Figure 2B; columns 4 and 5). An Ndc80 homologue has been purified with the SPB preparations from *S. cerevisiae*,; therefore, we looked to see whether the *S. pombe* protein colocalized with Cut12p, a protein that resides near the inner face of the SPB, adjacent to the nucleus (Osborne *et al.*, 1994; Bridge *et al.*, 1998; Wigge *et al.*, 1998; Wigge and Kilmartin, 2001). Ndc80p and Cut12p signals colocalized to some extent in all interphase cells (Figure 3, C and D), whereas the Cut12 signal was not clearly separable from that of Ndc80; it localized peripherally to Ndc80 relative to the 4′,6-diamidino-2-phenylindole (DAPI)-stained chromosomes (blue arc) in 83% of cells (N = 50; Figure 2D, columns 2, 4, 5, and 7). Because the Ndc80 signal also overlapped with Cnp1 we concluded that Ndc80 occupies a

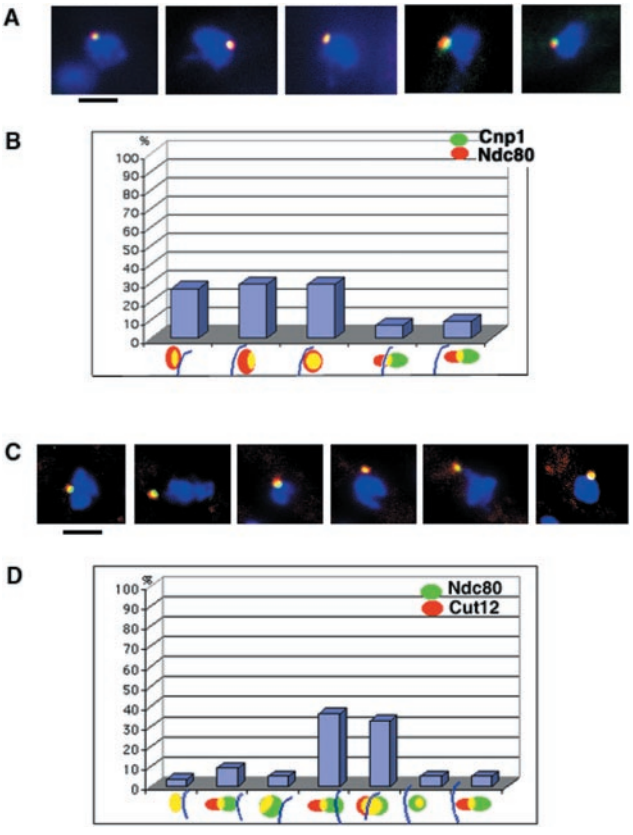

Figure 2. Ndc80 localizes with Cnp1 domain and extends toward the SPB. (A) IF pictures showing the localization of Cnp1 (green) and Ndc80 (red) proteins in interphase nuclei. Cells expressing Ndc80-GFP were stained with anti-GFP and anti-Cnp1 antibodies. (C) IF pictures showing the relative localization of Ndc80-GFP (green) and Cut12-Pk (red) proteins in interphase nuclei. Cells harboring the Cut12-Pk protein fusion were stained with anti-Pk and anti-Cnp1 antibodies (Mellone and Allshire, unpublished data). (A and C) DAPI (blue) was used to stain the DNA. Bar, 2.0 μm. (B and D) Statistical analysis of the relative positions of the markers as indicated in Figures B and D (n = 50 for each graph). Yellow color indicates colocalization in the merged picture and the blue arc indicates the position of the DAPI-stained chromatin.

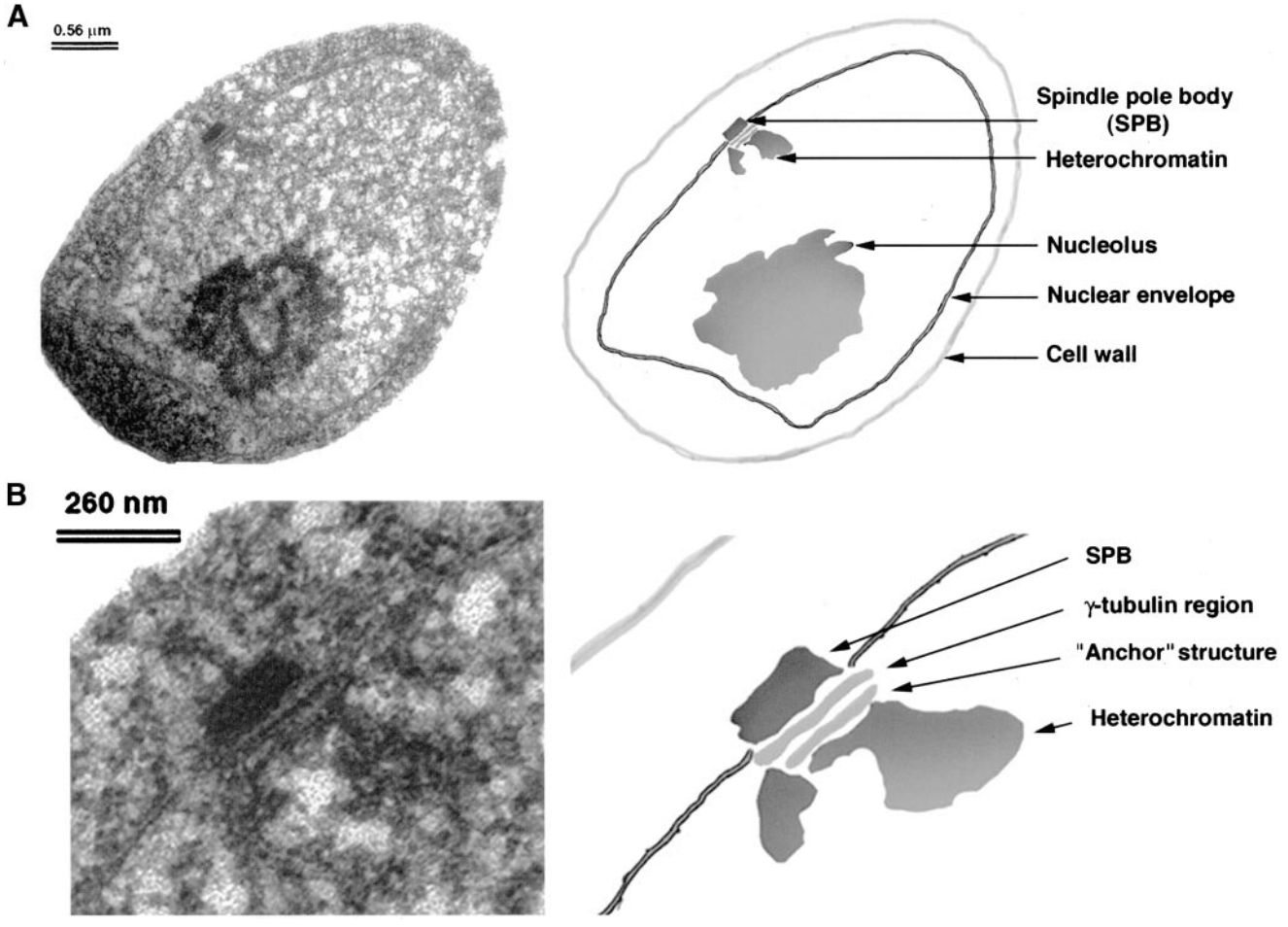

Figure 3. EM analysis of *S. pombe* centromeres. (A) A low-magnification EM micrograph of a section through a HPF-fixed and K11M-embedded interphase *S. pombe* cell. The cellular structures are indicated: cell wall, nuclear envelope, nucleolus, heterochromatin region, and SPB. Bar, 0.56 μm. (B) A higher magnification of the same cell. The nuclear structures indicated are SPB γ-tubulin region, anchor structure, and heterochromatin. Bar, 260 nm

position between the central core (Cnp1p) and the nuclear face of the SPB (Cut12p). Taken together these results indicated that there may be at least three distinct layers in the centromeres of *S. pombe*. Moreover, the position of the centromere appears to be dynamic in interphase cells. It was, however, difficult to resolve the structural components of the kinetochore by light microscopy.

EM Analysis of Centromeric Heterochromatin

To obtain more detailed information about fission yeast centromeres, log-phase cultures of *S. pombe* were immobilized by high pressure freezing (HPF), fixed by freeze substitution, embedded, and analyzed by EM. With this procedure the structures of nuclei and microtubules were generally well preserved. In addition to the nucleolus, the nucleus, containing another structure that stained more darkly than the surrounding nucleoplasm, was observed near the SPB (Figure 3). This structure was 200–300 nm wide and amorphous but generally of round shape. Based on its staining and its intranuclear position near the SPB, we inferred that this structure was centromeric heterochromatin (see below). At higher magnification we noticed another plate-like structure, ~250 nm wide and 20 nm thick, lying between the presumed centromeric heterochromatin and the previously described osmiophilic region where γ-tubulin is bound to the inner surface of the nuclear envelope, just opposite the SPB (Figure 3B; Ding *et al.*, 1997). We will refer to this new structure as the centromere "anchor," because it appears to base the centromeric heterochromatin near the γ-tubulin region that lies proximal to the nuclear envelope.

Fission yeast centromeres have previously been shown to cluster in close to the SPB (Funabiki *et al.*, 1993). To confirm the identity of the electron-dense material as centromeric heterochromatin, we carried out immuno-EM with antibodies that recognized the Swi6 component of this material. Antibodies against GFP were used to detect the GFP-Swi6 fusion protein (Pidoux *et al.*, 2000), because these were known to perform well in immuno-EM (Zeng *et al.*, 1999).

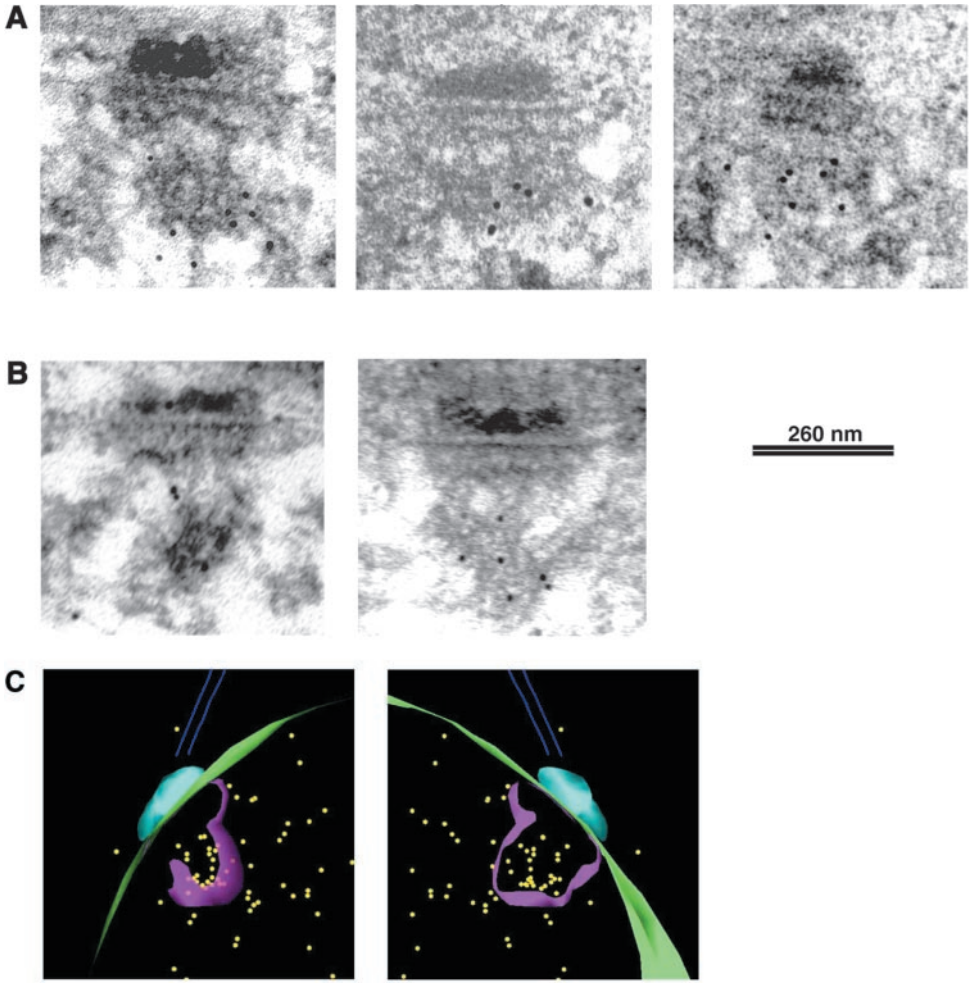

Figure 4. Swi6 is part of the heterochromatic domain, as detected by staining with antibodies and Au_{10}. (A) Representative micrographs showing Swi6 localization in G2 cells with single SPBs. (B) Swi6 immuno-EM picture showing parts of nuclei from late interphase or prophase cells with duplicated SPB. (C) Different angular views of a 3-D model showing the GFP-Swi6 immuno-EM localization (yellow). The increased intranuclear background labeling in the 3-D model compared with the Swi6 IF localization (Figure 1) is most likely caused by loss of Swi6 antigen during the IF processing because the diffuse haze of Swi6 is also observed by live analysis of GFP-Swi6 (Pidoux *et al.* 2000). The positions of the nuclear envelope (green), the SPB (light blue), cytoplasmic microtubules (dark blue), and the boundary of the heterochromatin domain (pink) are indicated.

The total number of gold particles (Au_{10}) corresponding to GFP-Swi6 on the heterochromatin structure was determined to compare whether the amount of Swi6 varied between the interphase (single SPB) and prophase (duplicated SPB) stages of the cell cycle (Figure 4, A and B; see MATERIALS AND METHODS). On average 5 ± 3 and 6 ± 2 Au_{10}, respectively, were bound to the each cross-section of the flanking domain at these two stages. Swi6 was present throughout the heterochromatin domain but not in the anchor structure. The heterochromatin domain and anchor structures persisted during the entire G2 phase of the cell cycle, because they were also apparent at prophase stages when the SPB was duplicated (Figure 4B).

3-D EM Analysis of the Central Core and Flanking Regions

The IF localizations of Swi6, Mis6 and Cnp1 described above indicated that the central core region of the centromere was often positioned differently from the flanking centromeric region. To determine the position of Swi6 within the 3-D structure of the interphase heterochromatin, cells carrying a GFP-Swi6 fusion were subjected to HPF, and serial sections of 30–50 nm were cut, stained with antibodies of interest, and imaged in the EM. Serial images of the labeled sections were aligned and 3-D models were constructed with the use of the IMOD software (see MATERIALS AND METHODS). In the resulting models GFP-Swi6Au_{10} was localized in the peripheral portion of the centromeric heterochromatin region, in some instances just outside the electron-dense region of that structure (Figure 4C).

To compare the position of Cnp1 with that of Swi6, cells carrying a GFP-Cnp1 fusion were prepared for immuno-EM and 3-D models were constructed as described above. In the resulting models GFP-Cnp1Au_{10} occupied a more central position within centromeric heterochromatin than that seen with Swi6p (compare with Figures 4 and 5); in some cases the Cnp1-GFP was adjacent to the anchor structure visible by EM (Figure 5, A, B, D, and E). Thus, Swi6 bound to centromere flanking region chromatin, *imr* and *otr*, is situated at the periphery of the clustered centromeric heterochromatin, whereas Cnp1, which binds to central core (*cnt*) region, is less abundant and localized centrally within the heterochromatin domain.

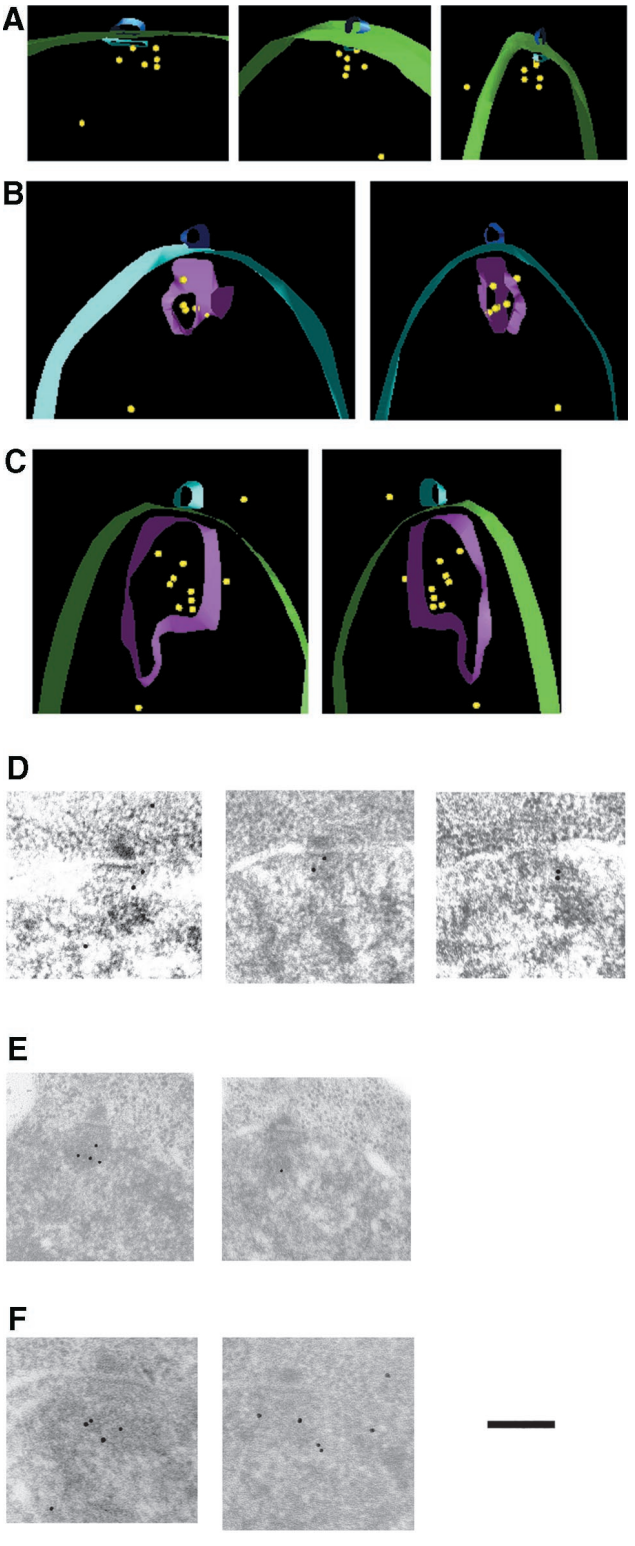

Figure 5. Cnp1 has a central localization within the heterochromatin domain. (A–C). Different angular views of 3-D models showing the GFP-Cnp1 immuno-EM localization (yellow). (A) The contours of the nuclear envelope (green) the SPB (light blue), and the anchor structure (dark blue) are indicated. (B) The contours of the nuclear envelope (green), the SPB (light blue), the anchor structure (dark blue), and the heterochromatin domain (pink) are indicated. (C) The contours of the nuclear envelope (green), the SPB (light blue), and the heterochromatin domain (pink) are indicated. (D–F) The primary micrographs representing the GFP-Cnp1 immuno-EM localization. (D) These three sections were used to create the 3-D model in A; (E) the two sections were used to create the 3-D model in B; and (D) the two sections were used to create the 3-D model in C. Bar, 260 nm.

Ndc80 Is Part of the Centromere Anchor Structure

Because the IF signals from Ndc80 localizes between Cnp1 and the SPB, we looked to see whether Ndc80 was part of the anchor structure. Immuno-EM was carried out as described above but with a strain expressing the Ndc80-GFP fusion (Wigge and Kilmartin, 2001). Some of the Ndc80 gold signal was located centrally within the heterochromatin domain, and some was indeed present on the anchor structure situated between the heterochromatin domain and the γ-tubulin region (Figure 6). The labeling density for Ndc80-GFP detected by anti-rabbit Au_{12} was 467 ± 103 on the 250-nm-wide and 20-nm-thick anchor structure and 44 ± 9 $Au_{12}/\mu m^2$ on the centromeric heterochromatin. The background in the nucleus and cytoplasm was 9 ± 6 and ≤1 $Au_{12}/\mu m^2$, respectively.

A Multilayered Organization of S. pombe *Centromeres*

To investigate how the distinct protein-binding domains within *S. pombe* centromeres corresponded to the observed structures, the distances from the nuclear face of the SPB to the gold particles representing the immuno-EM positions of Ndc80, Cnp1, and Swi6 signals were measured. The average distance to all the Ndc80 gold was 142 ± 106 nm (n = 49); to the Cnp1 gold it was 213 ± 59 nm (n = 37); and to the Swi6 gold it was 278 ± 65 nm (n = 58). The distributions of all these distances are shown graphically in Figure 7 A. A statistical analysis of these distributions of markers suggested that the corresponding proteins occupied significantly different positions relative to the SPB ($p < 0.00001$). It follows that they occupy different positions within the centromere structure. Only the Ndc80 protein was detected (and not Cnp1 or Swi6) within the region most proximal to the SPB (40–80 nm) corresponding to the anchor structure. Furthermore, within the heterochromatic domain most distal to the rest of the chromatin, 350–450 nm from the SPB, only Swi6 protein was present (and not Cnp1 or Ndc80). The distribution of Cnp1 peaks between that of Swi6 and Ndc80, consistent with the central position occupied by Cnp1 with the 3-D models in Figure 4C and Figure 5. Therefore, as indicated in the schematic model (Figure 7B) we concluded that the central core (Cnp1), the flanking centromere region (Swi6), and the anchor structure (Ndc80) occupy distinct layers. These probably correspond to different domains within the *S. pombe* centromere.

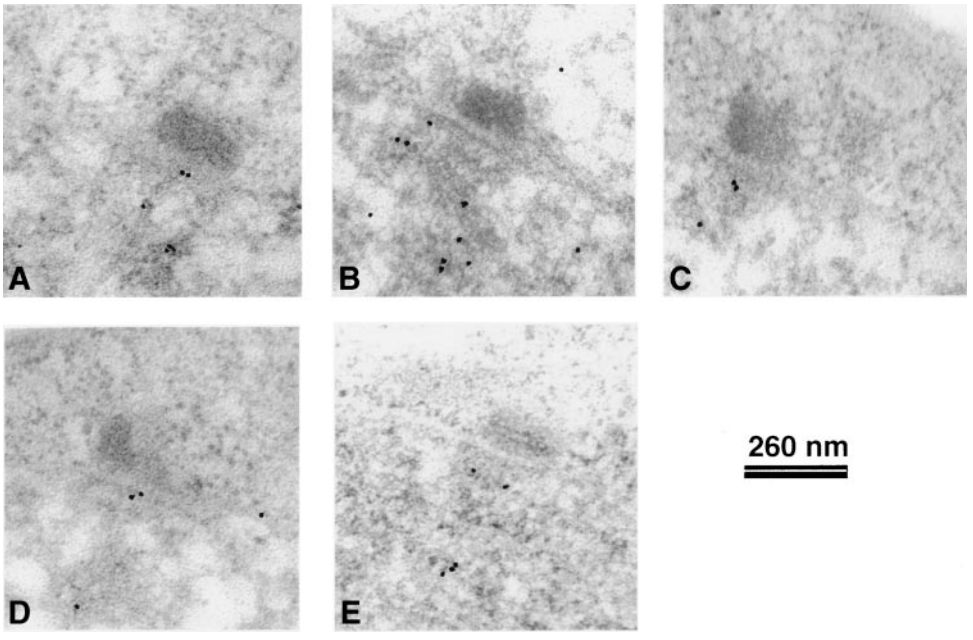

Figure 6. Ndc80 is part of the anchor domain. (A–E) Representative micrographs showing Ndc80-GFP immunolocalization in nuclei of G2 cells with single SPBs. Anti-rabbit-Au_{12} was used to detect Ndc80-GFP.

DISCUSSION

A Centromere Domain Structure Is Conserved from S. pombe *to Human*

In this work we have obtained evidence for a structure in the centromeric heterochromatin and the associated chromatin-SPB anchor in *S. pombe* cells at different stages of interphase. Although there is little precedence for the direct visualization of distinct chromosomal domains in yeast cells, these structures can now be compared with the equivalent structures in human cells.

Specimen preparation by HPF and subsequent analysis by EM have resulted in a slightly modified view of the human metaphase kinetochore structure, compared with that seen by conventional EM (McEwen *et al.*, 1998). Nonetheless, the basic organization of these centromere-associated structures was the same as that seen by conventional fixation, with an outer region (the plate-like structures) being distinct from the underlying heterochromatin. With the use of HPF and EM techniques on *S. pombe* cells we have found a striking similarity between structure of the interphase centromere structure and the metaphase centromere of humans. It is not clear why *S. pombe* centromeres are organized in this manner throughout G2, because the mammalian centromeres seem to unfold in S phase and then refold during G2 to reappear as typical kinetochore structures in late G2 (He and Brinkley, 1996). It could perhaps be a reflection of the shorter *S. pombe* cell cycles allowing approximately one order of magnitude less time for unfolding to occur. Another possibility is that maintenance of the connection between centromeres and the SPB serves a specific function in maintaining the *S. pombe* chromatin organization during G2.

It is interesting to note that the positions of some CENPs are conserved within the multilayered kinetochore structures from *S. pombe* to human. First we show that Cnp1 (*S. pombe* CENP-A) occupies a central position within interphase kinetochores distinct from Swi6. Recent studies, including live analysis, indicate that, although the human kinetochore unfolds and refolds during interphase, the human prekinetochore interphase structure remains ordered in interphase so that CENP-A localization is limited to the edge of a larger CENP-B heterochromatin domain even before the typical double dot structure appears in G2 (Pudenko *et al.*, 1997; Sugimoto *et al.*, 2000). At metaphase CENP-A is a component of the inner plate in human centromere (Vafa and Sullivan, 1997). Second, the more peripheral position of chromodomain protein Swi6 is reminiscent of the localization of HP1 to the underlying heterochromatin in humans. A third parallel is the localization of Ndc80 to the anchor structure in *S. pombe,* whereas the human homologue of Ndc80, HEC, is localized to the outer part of HeLa cell centromeres (Wigge and Kilmartin, 2001). It is possible that this conservation of position reflects centromere functions that are conserved across a broad range of eukaryotes. Because the anchor structure is adjacent to the γ-tubulin region from which microtubules are nucleated in mitosis, the anchor structure may carry out a function in mitosis analogous to the outer plate structure in human centromeres, perhaps harboring microtubule interactions as discussed by Wigge and Kilmartin (2001). The *S. pombe* central core and the human inner plate could have a similar function. Interestingly, cells with a central core marker *mis6–302* temperature-sensitive allele show both defective interphase centromere clustering and a mitotic missegregation defect of chromosomes, which is consistent with a biorientation defect of sister kinetochores (Saitoh *et al.*, 1997). Mis6 is required to recruit Cnp1 to the central core (Takahashi *et al.*, 2000). Hence, it is possible that Mis6- and Cnp1-mediated clustering is a prerequisite for correct biorientation and that biorientation is the conserved function for the inner plate. Based on their position within the structure it is also conceivable that the flanking region and the underlying heterochromatin in human centromere have similar functions. *S. pombe* cells with

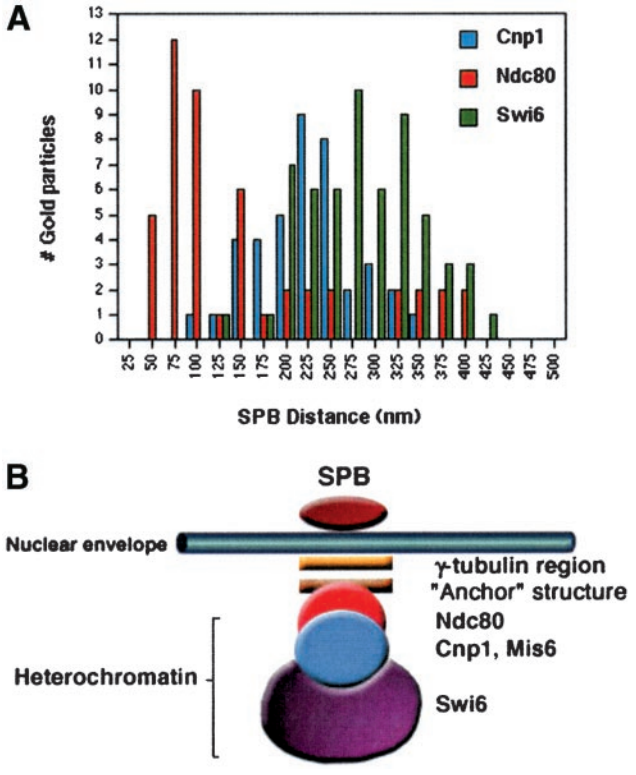

Figure 7. *S. pombe* centromeres are multilayered. (A) Histogram showing the distribution of SPB-gold distances for Ndc80, Cnp1, and Swi6 proteins in interphase cells. (B) Schematic model of *S. pombe* centromere cluster in interphase cells. The distribution of Ndc80, Cnp1, and Swi6 proteins are indicated in relation to the observed centromeric anchor and heterochromatin structures.

mutations that disturb the integrity of the flanking regions such as *swi6, rik1, clr4, and csp7-12* display a typical lagging chromosome phenotype in anaphase, but interphase clustering of centromeres is normal (Ekwall *et al.*, 1996, 1999).We are hopeful that this work will open the way for detailed EM studies of the putative defective centromere structures corresponding to the distinct functional defects in these mutants.

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